AD_____

GRANT NO: DAMD17-94-J-4347

TITLE: Engineering Bispecific Antibodies That Target ERBB-2 on Breast Cancer Cells

PRINCIPAL INVESTIGATOR(S): Doctor David M. Kranz

CONTRACTING ORGANIZATION: University of Illinois
Champaign, Illinois 61820

REPORT DATE: September 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1995	Annual 1 Sep 94 - 31 Aug 95

4. TITLE AND SUBTITLE
Engineering Bispecific Antibodies that Target ERBB-2 on Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-94-J-4347

6. AUTHOR(S)
Dr. David M. Kranz

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois
Champaign, Illinois 61820

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The goals of this project are to construct single-chain bispecific antibodies that target erbB-2 on breast cancer cells, to evaluate the susceptibility of the tumor cells to lysis mediated by bispecific antibodies and cytotoxic T cells (CTL), and to develop an animal model that can evaluate the in vivo effectiveness of these agents. The following aims were accomplished during the past year. 1) V region genes that encode the anti-erbB-2 antibody 800E6 were cloned by PCR and expressed as a single-chain Fv in E. coli. The scFV-800E6 had an affinity for erbB-2 that was nearly identical to the parental antibody. 2) Evaluation of various human breast cancer lines for their ability to be lysed by CTL showed that there is a wide range of intrinsic susceptibilities. The most resistent tumor, SKBR3, was considerably more susceptible to killing when incubated with a combination of TNF-α and IFN-γ. 3) Progress was made on establishing a colony of immunodeficient mice that contain an endogenous population of CTL for in vivo targeting of transplanted human tumors. These mice express mouse TCR transgenes but have the recombinational activation gene (RAG-1) knocked out.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Bispecific Antibodies/erbB-2/T cells/Transgeneic mice breast cancer	28
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	PR	- Project
G	- Grant	TA	- Task
PE	- Program Element	WU	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

DOD	- See DoDD 5230.24, "Distribution Statements on Technical Documents."
DOE	- See authorities.
NASA	- See Handbook NHB 2200.2.
NTIS	- Leave blank.

Block 12b. Distribution Code.

DOD	- Leave blank.
DOE	- Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
NASA	- Leave blank.
NTIS	- Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

*U.S.GPO:1993-0-358-779

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

X Where copyrighted material is quoted, permission has been obtained to use such material.

X Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

David M. Kuny 9/22/95

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION
(Adapted in part from original proposal)

Successful treatment of breast cancer requires the identification of specific targets for the rational design of therapeutic agents. One such target, described several years ago, is the oncogene product erbB-2 (1-3). This protein is expressed on the surface of tumor cells in approximately 30% of women with the poorest prognosis for survival (4). The expression of erbB-2 does not appear to be correlated, either positively or negatively, with the estrogen receptor (5), although a recent report suggests that the erbB-2 signalling pathway targets the estrogen receptor resulting in estrogen-independent growth of tumor cells (6). However, it's expression is associated with an increase in the resistance of tumor cells to lysis by some natural immune mechanisms such as tumor necrosis factor and lymphokine activated killer cells (7,8).

A number of investigators have now begun to use antibodies specific for erbB-2 as possible therapeutic agents (9-16). The agents include immunotoxins, radioimmunoconjugates, and bispecific antibodies. The latter antibodies are intended to mediate effects by directing the lysis of tumor cells through cytotoxic effector cells. One of the predicted advantages of bispecific antibodies is that they should not have side effects associated with delivery of toxins or isotopes. Bispecific antibodies have in fact begun to be examined in several clinical trials, with some encouraging results.

Despite their promise and emergence into clinical trials, there are many questions that need to be addressed before optimal uses, with minimal side effects, of bispecific antibodies can be realized. For example, genetic engineering now provides a method for constructing smaller, potentially more stable, antibodies. Are these antibodies likely to be more effective than conventional intact antibodies? Will the natural resistance of erbB-2$^+$ tumors cells present an obstacle to successful bispecific antibody therapy? Can a system that uses a patients own immune cells be developed so that *ex vivo* activation of effector cells is not needed? This would obviously allow the treatment of a much larger patient base than would be possible if effector cells must be cultured for every patient.

It would clearly be useful to have a system that could provide answers to these questions in order to design the most effective clinical trials of bispecific antibodies. There has been no animal model developed that can evaluate all of these issues using human breast cancer cells. The purpose of this project is twofold. First, to construct novel bispecific antibodies that can be expressed as a single-chain in *E. coli* (17). Second, to develop an *in vivo* animal model that will allow the testing of these agents in comparison with other conventional bispecific antibodies. There are many potential therapeutic regimens that will need to be evaluated. To do so, an animal model that will not require introduction of human effector cells and that will most resemble the situation that will be encountered in the human disease will be developed. It is anticipated that these animals could also serve as models for immune modulating agents that are developed by other laboratories.

The methods used to approach these goals involve many reagents and strains of mice that have been developed in my lab and in collaboration with others over the past ten years. The general strategy involves the use of the following: 1) Monoclonal antibodies and single-chain antibodies that are specific for the T cell receptor of a mouse cytotoxic T lymphocyte clone 2C (18); 2) Monoclonal antibodies that are specific for human erbB-2, provided by Pier Natali (11); 3) Cloning and expression systems for the overproduction of single-chain monospecific and bispecific antibodies (17,18); 4) Human breast cancer tumor cell lines obtained from the ATCC; 5) Transgenic mice that express the TCR from CTL clone 2C (19,20); and 6) RAG-1 knockout mice, provided by Susumu Tonegawa's lab, that lack B and T cells because of the absence of recombinational-activation-gene 1 (21).

The four specific aims of the project are: 1) Following a strategy that has recently proven successful in our lab, to engineer a single-chain bispecific antibody to erbB-2 and the TCR of a mouse CTL clone; 2) To use a simple screening method to search for agents that increase the sensitivity of erbB-2^+ breast cancer cell lines to lysis by CTL. 3) To develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, TCR transgenic mice (TCR/recombination-activating-gene knockouts, RAG-2^-). 4) To test the *in vivo* effectiveness of various bispecific antibody regimens in the TCR/RAG-2^- human xenograft system. Specific details concerning progress in the first year of this award toward these goals are provided in the body of this report, below.

BODY

Progress has been made toward various aspects of three out of the four specific aims (1 - 3). Methods, results, and a brief discussion are presented within each aim:

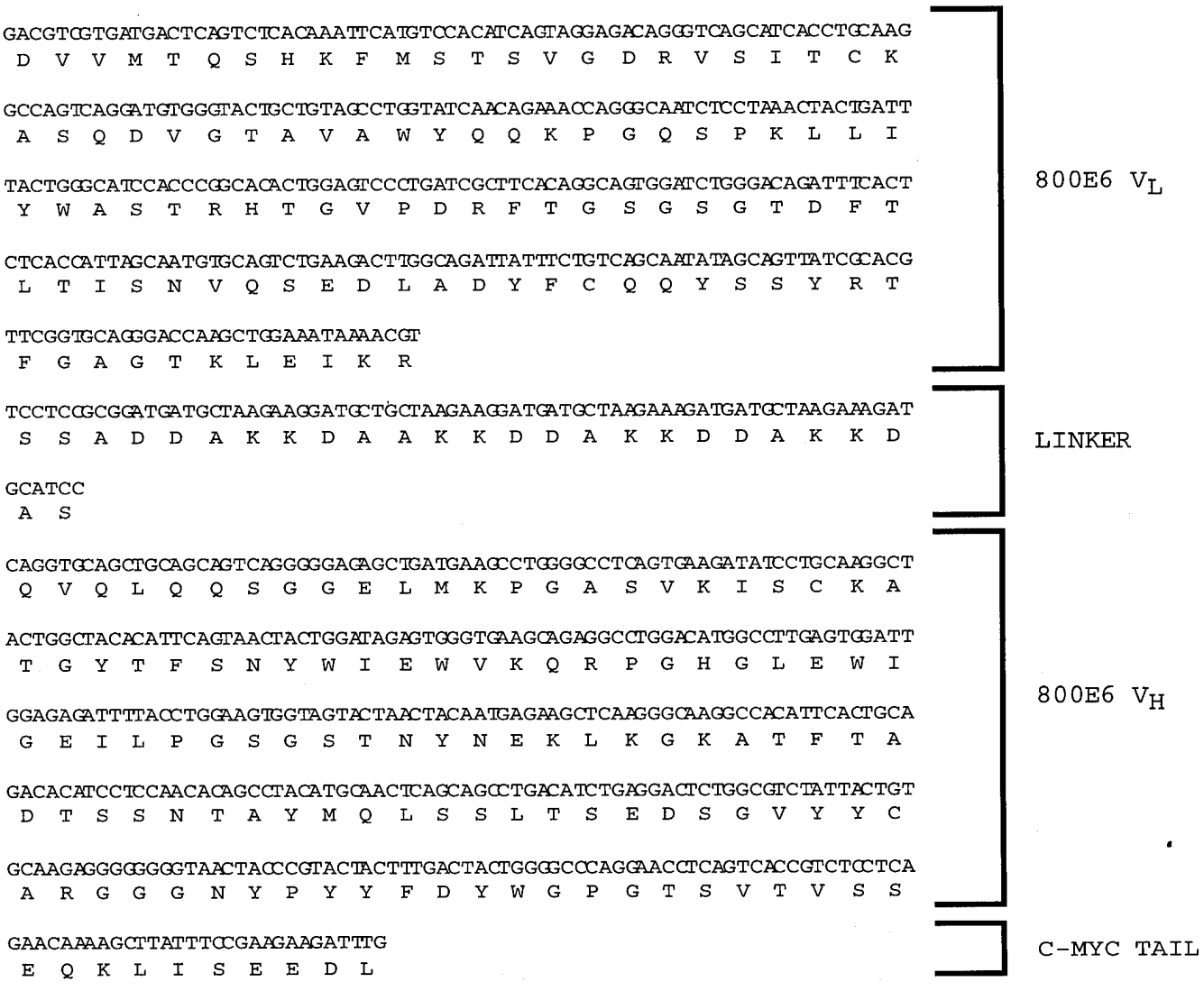
Specific Aim 1. Construct and characterize a single-chain bispecific antibody (anti-erbB-2/anti-TCR, called $scFv_2$).

Cloning of the V_H and V_L genes from the anti-erbB-2 antibody 800E6. Two different mouse hybridomas (900F4 and 800E6) that secrete antibodies with high affinity for human erbB2 were used to clone a single-chain anti-erbB2 antibody. RNA derived from each hybridoma was isolated and used in the synthesis of cDNA. Degenerate primer pairs for mouse V_L and J_L and V_H and J_H were used to amplify directly from cDNA following a procedure that has been used successfully by our lab with other antibodies (22). Both hybridomas yielded PCR products of the expected size (~350 bp) for the V_H region but both hybridomas yielded only a 230 bp product for the V_L region (in contrast to the ~ 350 bp that was expected). It is likely that both antibodies use V_L region genes that were not represented by the degenerate primers. Because we were able to successfully clone the V_H PCR product for 800E6 initially, the light chain from 800E6 was isolated from SDS-PAGE gels in an effort to obtain information about the V_L gene family used by this antibody. The sequence DIVMXQXHKF was identified and this sequence

corresponded to the V_K subgroup I family in mice. A primer that corresponded to this sequence was used to successfully amplify a 350 bp PCR product from the 800E6 cDNA.

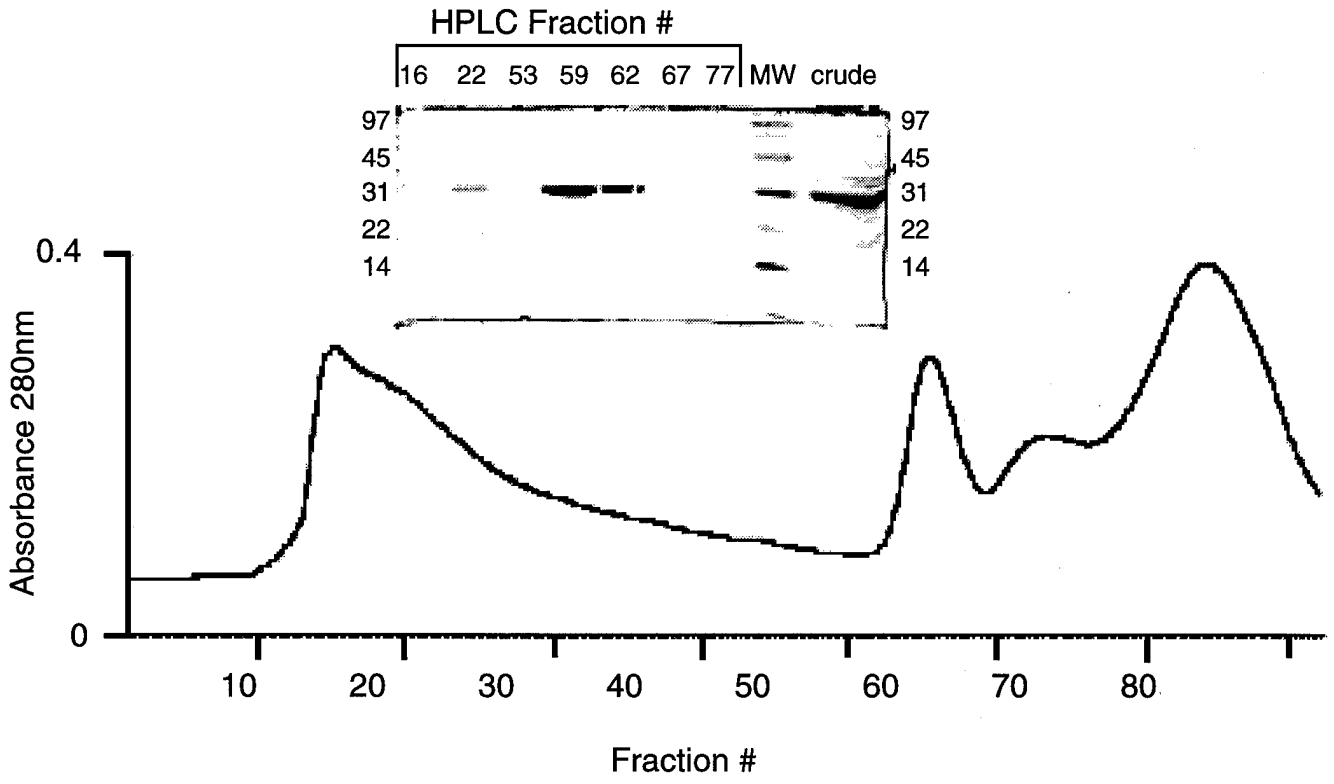
The V_H and V_L PCR products from 800E6 were cloned sequentially into the single-chain expression vector that we have used previously (18). The nucleotide sequence of the 800 base pair insert was determined by automated sequencing with primers that flank the scFv gene (Figure 1). Both the V_L and V_H genes contained all of the conserved residues that are characteristic of Igs. These included the canonical FGXG of J_L and WGXG of J_H and two cysteines that form the basis of the typical Ig fold. The construction was also shown to be expressed at the protein level based on its reactivity with a monoclonal antibody that is specific for the c-myc peptide that was included at the carboxy terminus of the scFv (data not shown).

Figure 1. Sequence of the anti-erbB2 scFv from hybridoma 800E6.



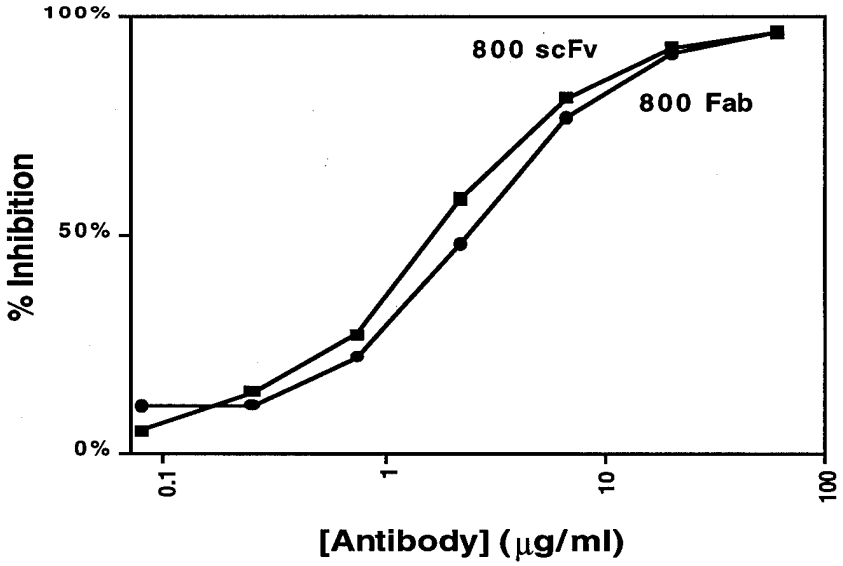
Expression and Purification of scFv-800E6. The anti-erbB2 scFv was expressed in *E. coli* in large scale fermentation, the cells were broken by passage through a microfluidizer, and the inclusion body pellets were solubilized in guanidine. After refolding by dialysis in a Tris-EDTA-arginine buffer (18), scFv was purified by size exclusion through a G200 HPLC column. Samples were analyzed by SDS-PAGE (Figure 2) and ELISA with the anti-c-myc antibody (data not shown). The major protein in the crude preparation exhibited a size of 31 kDa, consistent with the predicted size of the scFv-800E6 (33,559 Da). Approximately 75% of this preparation migrated as aggregates in the size exclusion column but a monomer peak of ~30 kDa (fractions 55 to 60) was identified by ELISA with anti-c-myc as the scFv-800E6. The anti-erbB2 scFv monomer could be isolated at ~1 mg/liter of bacterial culture.

Figure 2. HPLC G200 gel filtration profile and SDS-PAGE of 800E6 scFv.



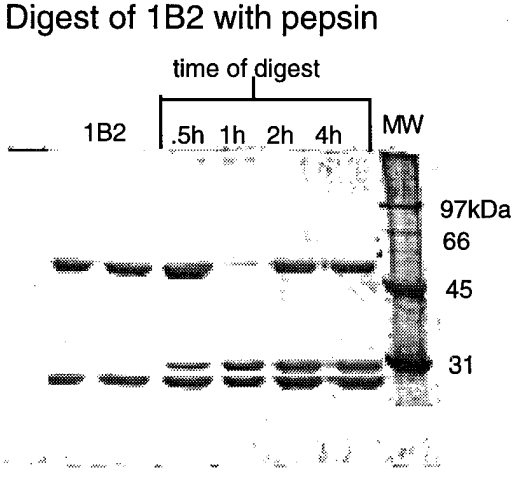
Cell Surface Binding of scFv-800E6 to erbB2. In order to determine if the scFv-800E6 could bind to erbB2 on the cell surface, two different assays were used. Initially, flow cytometry with the erbB2 positive tumor cell line SKBR3 was performed in the presence of the scFv, followed by anti-c-myc, and then fluorescein-labelled anti-mouse Ig. The result showed that the scFv preparation bound specifically to the SKBR3 cells (data not shown). In order to determine the affinity of the recombinant scFv relative to the intact antibody, a competitive inhibition assay was performed with ^{125}I-labelled Fab fragments derived from 800E6. SKBR3 cells were incubated with a constant amount of ^{125}I-Fab in the presence of various concentrations of unlabeled scFv or Fab fragments (Figure 3). The result indicated that the scFv has an affinity that is within two-fold of that of the Fab fragment ($K_D \sim 10^{-9}$ M). In addition, the scFv preparation appears to be predominantly in the properly folded form.

Figure 3. Comparison of erbB2 binding by 800E6 Fab fragments and scFv. ^{125}I-Fab fragments were incubated with the erbB2 positive tumor cell SKBR3 in the presence of the indicated concentration of inhibitor. After 1 hour on ice, the mixture was centrifuged through a layer of oil to separate bound and free ^{125}-I-Fab.



Preparation of Fab_2 from 800E6 and anti-TCR antibody 1B2. In order to eventually evaluate the effectiveness of the bispecific single-chain antibodies, it will be necessary to compare them with antibodies prepared by standard chemical-linkage technology. Toward this effort, the 800E6 anti-erbB2 antibody and the anti-TCR antibody 1B2 will be linked by preparing $(Fab)'_2$ fragments of each, followed by linkage through disulfide bonds (23). Although both 800E6 and 1B2 can be readily cleaved with papain to yield Fab fragments (see above and ref 18), we have found that pepsin digestion to produce Fab_2 fragments is more problematic. Nevertheless, by varying the pH and time of the reaction (24), we have identified conditions that yield ~20 to 50% Fab_2 of 1B2 (Figure 4) and 800E6 (data not shown). The Fab_2 fragments will be purified by G200 HPLC and the bispecific antibody prepared as previously described (23).

Figure 4. SDS-PAGE gel of reduced Fab_2 fragments from anti-TCR antibody 1B2. The 30 kDa band corresponds to the amino terminal region of the H chain after pepsin cleavage.



Specific Aim 2. Search for agents that increase the sensitivity of erbB-2$^+$ breast cancer cell lines to lysis by cytotoxic T lymphocytes.

Susceptibility of breast cancer cell lines to lysis mediated by bispecific antibodies and CTL clone 2C. Previous studies have shown that erbB2 positive tumor cells are relatively resistant to lysis by TNF (8) and that tumor cells vary in their susceptibility to direct growth inhibition by anti-erbB2 antibodies (12). To begin to evaluate their susceptibility to CTL-mediated lysis in the presence of bispecific antibodies, various breast cancer lines were used as target cells in a cytotoxicity assay with the mouse CTL clone 2C. This clone expresses the TCR that will be used to redirect the lysis of tumor cells by CTL *in vivo* (Specific Aim 3 and 4). In order to evaluate intrinsic susceptibility to the lytic mechanism, target cells were labeled with fluorescein to the same density and assayed in the presence of an anti-fluorescein bispecific single chain antibody (17).

As shown in Table 1, human tumor cell lines varied considerably in their susceptibility. The erbB2 levels, in mean fluorescence units, of these lines were shown by flow cytometry to be: SKBR3 (150) > MB-453 (106) > MCF7 (15) > MB-468 = Daudi (0). Daudi is a human lymphoblastoid line that we have previously shown is susceptible to lysis by 2C (17).

Table 1. Susceptibility of breast cancer cell lines to lysis mediated by bispecific antibodies and CTL clone 2C.

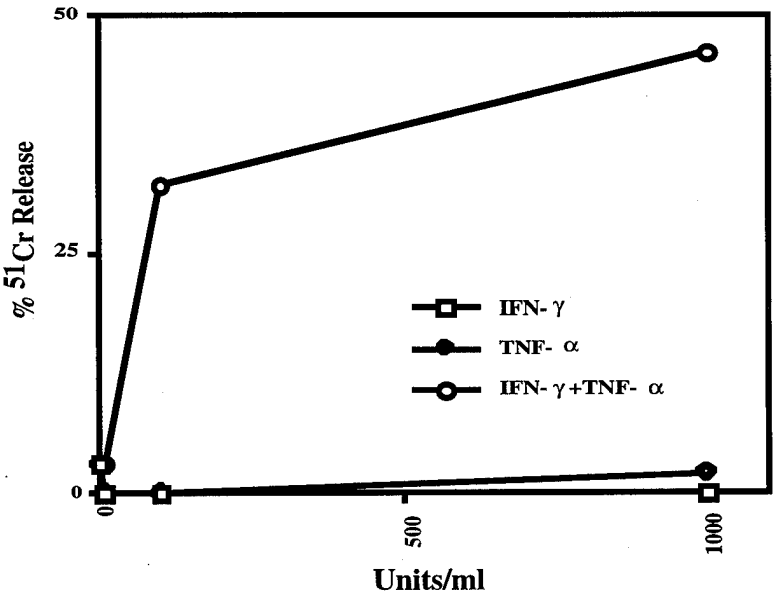
	[scFv2] µg/ml	E:T Ratio	Cell Line	% Specific Lysis
Experiment 1	18	6:1	Daudi	24
			SK-BR-3	1
			MCF-7	3
			MDA-MB-453	4
			MDA-MB-468	14
Experiment 2	50	6:1	Daudi	82
			MDA-MB-453	32
Experiment 3	50	30:1	Daudi	64
			MDA-MB-453	27
Experiment 4	25	10:1	Daudi	54
			SK-BR-3	8
Experiment 5	50	10:1	Daudi	54
			SK-BR-3	8
			MCF-7	6
			MDA-MB-453	36
			MDA-MB-468	42
			T-47-D	20

The intrinsic susceptibility of the tumor lines did not correlate directly with erbB-2 level or with the level of Fas as measured by flow cytometry with the anti-Fas monoclonal antibody UB2. However, SKBR3 which has the highest level of erbB2, has always shown the most resistance to lysis by CTL 2C. Obviously, if some primary tumors exhibit similar levels of resistance to lysis, it will be necessary to either evaluate susceptibility prior to treatment or to identify agents that will increase susceptibility.

Effects of pretreatment of breast cancer cell lines with anti-erbB-2 antibodies. A previous report has demonstrated that anti-erbB2 antibodies can increase the susceptibility of tumor cells to treatment with cis-diamminedichloroplatinum (10). To determine if anti-erbB2 treatment might increase susceptibility to CTL lytic mechanisms, SKBR was treated for various lengths of time with the anti-erbB2 antibodies 800E6 and 900F4. Treated and untreated cells were fluorescein labeled and assayed as target cells in a cytotoxicity assay with bispecific antibody and CTL 2C as described above. Susceptibility of the SKBR3 lined was not increased under any conditions (varying times, concentrations of antibodies, or mixtures of the antibodies).

Effects of pretreatment of breast cancer cell lines with the cytokines IFN-γ and TNF-α. Cytokines can alter the susceptibility of tumor cells to cell-mediated lysis (25) and more specifically, IFN-γ has been shown to increase the susceptibility of erbB-2+ tumor lines to LAK-mediated lysis (26). To examine the effect of cytokines on CTL-mediated lysis of SKBR3, TNF and IFN were tested separately or in combination by treatment of SKBR3 for 2 days prior to a cytotoxicity assay, as described above. As shown in Figure 5, treatment with the combination yielded a dramatic increase in susceptibility to lysis by CTL 2C. The effect required 24 to 48 hours of treatment and it was clearly due to the synergistic action of these cytokines on SKBR3 and not on the CTL (data not shown). The mechanism of action of this finding is not clear at this time, but it does not appear to be due to an increase in the level of Fas on the surface of SKBR3. In addition the effect appears to vary among the different tumor cell lines (i.e. increased susceptibility was observed for some but not all of the lines tested; data not shown).

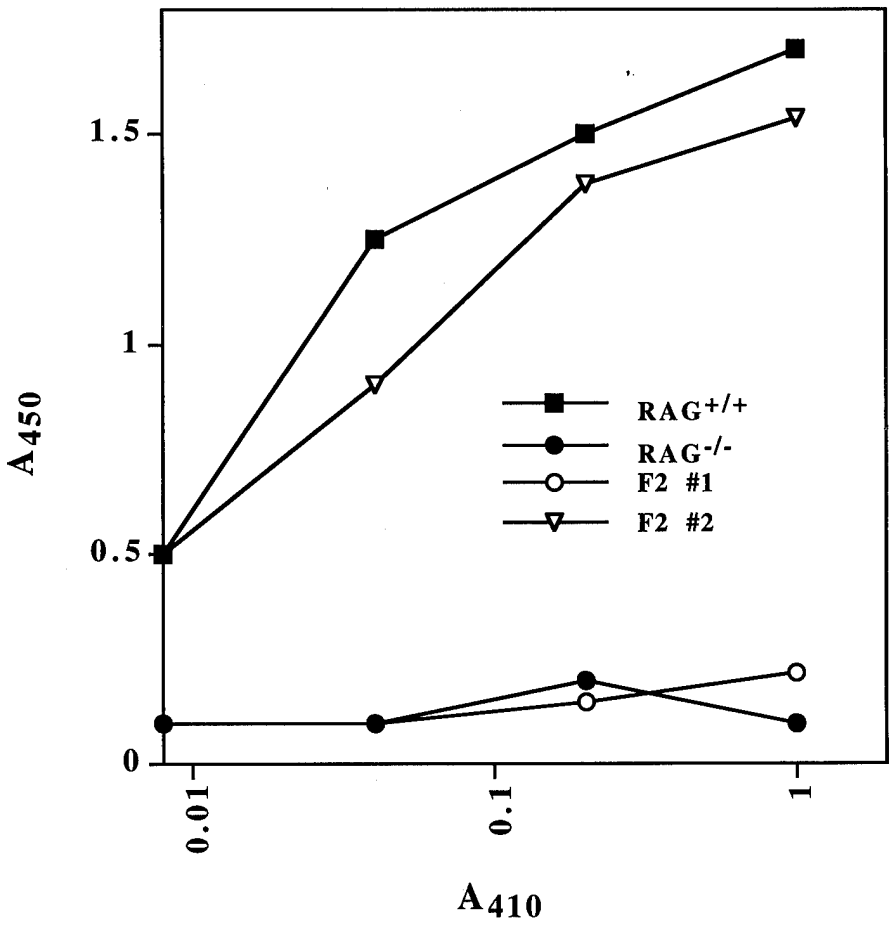
Figure 5. Increased susceptibility of SKBR tumor cells to CTL-mediated lysis after treatment with a combination of IFN-γ and TNF-α.



Specific Aim 3. Develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, T cell receptor transgenic mice.

Breeding, maintenance, and testing of immunodeficient, T cell receptor transgenic mice. Immunodeficient TCR/RAG-2 were to be provided by Dennis Loh's lab after they had successfully produced and screened progeny of the 2C TCR x RAG-2 knockout mice (19, 27). Dennis Loh recently moved to industry and these mice were not available. However, Dennis provided us with the 2C TCR breeding pairs and immunodeficient RAG-1 knockout mice (21) were obtained from Jackson Labs. We have established colonies of each of these mice and now have approximately 40 mice of the TCR x RAG F_2 generation. Approximately 1/4 of these will be $RAG^{-/-}$ and of these 1/4 should be homozygous for the TCR transgene. Because serum IgM is not passed maternally to the fetus (28), an ELISA that is specific for IgM was used to screen sera obtained from the tail vein of mice (representative experiment shown in Figure 6). Seven of the 40 mice were identified as Ig negative and thus as $RAG^{-/-}$. Of these, four mice contained peripheral lymphocytes as detected by immunohistological staining and thus have been tentatively identified as either $TCR^{+/-}RAG^{-/-}$ or $TCR^{+/+}RAG^{-/-}$. These mice have now been mated in order to generate a continuous colony of $TCR^{+/+}RAG^{-/-}$. We anticipate having a colony of mice for transplantation studies, as further outlined in Specific Aim 3, in 6 to 8 months.

Figure 6. ELISA of serum IgM from $RAG^{-/-}$, $RAG^{+/+}$, and two F2 mice. A_{450} refers to the IgM level and A_{410} refers to the hemoglobin level and thus amount of blood.



CONCLUSIONS

During the first year of this grant, each of the tasks shown below with an * were completed and significant progress was made on tasks indicated with an **:

***Task 1,** Cloning, expression, and testing of erbB-2 single-chain antibody, months 1-12.

***Task 2,** Screening of tumor cell lines for susceptibility to CTL-mediated lysis using the anti-fluorescein bispecific antibody, months 1-12.

****Task 3,** Breeding of transgenic TCR/RAG-2$^-$ mice and testing of peripheral blood T cells for reactivity with 1B2 antibodies, months 1-20. It is anticipated that approximately 175 mice will be produced by the end of this period.

Task 4, Cloning, expression, and *in vitro* testing of bispecific single-chain $scFv_2$ antibody (1B2/erbB-2), months 13-24.

****Task 5,** Screening of tumor cell lines for increased susceptibility to CTL-mediated lysis when tumor cells are treated with: anti-erbB-2 antibodies, IFN-γ, TNF-α, estrogen, tamoxifen, months 13-30.

Task 6, Transplantation of various erbB-2$^+$ tumor cell lines into TCR/RAG-2$^-$ mice and evaluation of tumor incidence, months 16-36. It is anticipated that approximately 15 mice per month will be used.

****Task 7,** Purification and *in vitro* testing of bispecific Fab_2 antibody (1B2/erbB-2), months 25-36.

Task 8, *In vivo* testing of bispecific antibodies in TCR/RAG-2- mice that have received human tumor transplants, months 30-48. It is anticipated that approximately 15 mice per month will be used.

Publications of the Principle Investigator During the Past Year

Kranz, D.M., M. Gruber, and E. Wilson. (1995) Properties of Bispecific Single-Chain Antibodies Expressed in *E. coli. J. Hematotherapy*, in press.

Kranz, D.M., T.A. Patrick, K.E. Brigle, M. J. Spinella, and E.J. Roy. (1995) Conjugates of Folate and Anti-T Cell Receptor Antibodies Specifically Target Folate receptor Positive Tumor Cells for Lysis. *Proc. Natl. Acad. Sci. USA* in press.

Cho, B.K., B. A. Schodin, and D.M. Kranz. (1995) Characterization of a Single-Chain Antibody to the β-Chain of the T Cell Receptor. *J. Biol. Chem.* in press.

Future Work:

During the next year, we intend to complete the tasks indicated above by an ** and to begin tasks 4 and 6.

Changes will include the testing of the erbB-2 positive ovarian tumor line SKOV3 for its ability to form tumors in RAG$^{-/-}$ mice. This line has been previously used as a transplantable

tumor into immunodeficient mice in erbB-2 targeting experiments (29). Once the conditions that allow this line to grow as a transplant have been determined, other erbB2$^+$ breast cancer cell lines will also be evaluated.

The SKOV3 line may have another advantage which we would like to pursue: 80% of ovarian tumors express on their surface another tumor-associated antigen called the folate receptor. We have recently shown that such folate receptor positive tumors can be targeted *in vitro* with conjugates of folate and an antibodies to the T cell receptor (30). Use of the SKOV3 cell line, or another line that we identify as both erbB-2$^+$ and folate receptor$^+$, may thus allow us the opportunity to test the efficacy of targeting two independently expressed tumor antigens with bispecific antibodies. It is reasonable to predict that this approach will be superior to targeting a single antigen because it may minimize the problems of tumor escape associated with the proliferation of tumor antigen negative variants.

REFERENCES

1. **Schechter, A. L., M.-C. Hung, L. Vaidyanathan, R. A. Weinberg, T. L. Yang-Feng, A. Ullrich, L. Coussens.** 1985. The *neu* Gene: An *erb*B-Homologous Gene Distinct from and Unlinked to the Gene Encoding the EGF Receptor. *Science.* 229:976-978.

2. **Bargmann, C. I., M.-C. Hung, and R. A. Weinberg.** 1986. The *neu* Oncogene Encodes an Epidermal Growth Factor Receptor-Related Protein. *Nature.* 319:226-230.

3. **King, C. R., M. H. Kraus, S. A. Aaronson.** 1985. Amplification of a Novel v-*erb*B-Related Gene in a Human Mammary Carcinoma. *Science.* 229:974-976.

4. **Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, W. L. McGuire.** 1987. Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/*neu* Oncogene. *Science.* 235:177-182.

5. **McCann, A. H., P. A. Dervan, M. O'Regan, M. B. Codd, W. J. Gullick, B. M. J. Tobin, and D. N. Carney.** 1991. Prognostic Significance of c-*erb*B-2 and Estrogen Receptor Status in Human Breast Cancer. *Cancer Res.* 51:3296-3303.

6. **Pietras, R. J., J. Arboleda, D. M. Reese, N. Wongvipat, M. D. Pegram, L. Ramos, C. M. Gorman, M. G. Parker, M. X. Sliwkowski, and D. J. Slamon.** 1995. HER-2 Tyrosine Kinase Pathway Targets Estrogen Receptor and Promotes Hormone-Independent Growth in Human Breast Cancer Cells. *Oncogene* 10:2435-2446.

7. **Hudziak, R. M., G. D. Lewis, M. R. Shalaby, T. E. Eessalu, B. B. Aggarwal, A. Ullrich, and H. M. Shepard.** 1988. Amplified Expression of the HER2/ERBB2 Oncogene Induces Resistance to Tumor Necrosis Factor a in NIH 3T3 Cells. *Proc. Natl. Acad. Sci. USA.* 85:5102-5106.

8. **Lichtenstein, A., J. Berenson, J. F. Gera, K. Waldburger, O. Martinez-Maza, and J. S. Berek.** 1990. Resistance of Human Ovarian Cancer Cells to Tumor Necrosis Factor and Lymphokine-activated Killer Cells: Correlation with Expression of HER2/*neu* Oncogenes. *Cancer Res.* 50:7364-7370.

9. **McKenzie, S. J., P. J. Marks, T. Lam, J. Morgan, D. L. Panicali, K. L. Trimpe, and W. P. Carney.** 1989. Generation and Characterization of Monoclonal Antibodies Specific for the Human *neu* Oncogene Product, p185. *Oncogene.* 4:543-548.

10. **Hancock, M. C., B. C. Langton, T. Chan, P. Toy, J. J. Monahan, R. P. Mischak, and L. K. Shawver.** 1991. A Monoclonal Antibody Against the c-*erb*B-2 Protein Enhances the Cytotoxicity of cis-Diamminedichloroplatinum against Human Breast and Ovarian Tumor Cell Lines. *Cancer Res.* 51:4575-4580.

11. **Digiesi, G., P. Giacomini, R. Fraioli, M. Mariani, M. R. Nicotra, O. Segatto, and P. G. Natali.** 1992. Production and Characterization of Murine mAbs to the Extracellular Domain of Human Neu Oncogene Product GP185^{HER2}. *Hybridoma.* 11:519-527.

12. **Lewis, G. D., I. Figari, B. Fendly, W. L. Wong, P. Carter, C. Gorman, H. M. Shepard.** 1993. Differential Responses of Human Tumor Cell Lines to Anti-p185^{HER2} Monoclonal Antibodies. *Cancer Immunol. Immunother.* 37:255-263.

13. **Adams, G. P., J. E. McCartney, M.-S. Tai, H. Oppermann, J. S. Huston, W. F. Stafford III, M. A. Bookman, I. Fand, L. L. Houston, and L. M. Weiner.** 1993. Highly Specific *in Vivo* Tumor Targeting by Monovalent and Divalent Forms of 741F8 Anti-c-*erb*B-2 Single-Chain Fv. *Cancer Res.* 53:4026-4034.

14. **De Santes, K., D. Slamon, S. K. Anderson, M. Shepard, B. Fendly, D. Maneval, and O. Press.** 1992. Radiolabeled Antibody Targeting of the HER-2/*neu* Oncoprotein. *Cancer Res.* 52:1916-1923.

15. **Shalaby, M. R., H. M. Shepard, L. Presta, M. L. Rodrigues, P. C. L. Beverley, M. Feldmann, and P. Carter.** 1992. Development of Humanized Bispecific Antibodies Reactive with Cytotoxic Lymphocytes and Tumor Cells Overexpressing the *HER2* Protooncogene. *J. Exp. Med.* 175:217-225.

16. **Weiner, L. M., M. Holmes, G. P. Adams, F. LaCreta, P. Watts, and I. Garcia de Palazzo.** 1993. A Human Tumor Xenograft Model of Therapy with a Bispecific Monoclonal Antibody Targeting c-*erb*B-2 and CD16. *Cancer Res.* 53:94-100.

17. **Gruber, M., B. Schodin, E. Wilson, and D. M. Kranz.** 1994. Efficient Tumor Cell Lysis Mediated by a Bispecific Single-Chain Antibody Expressed in *E. coli*. *J. Immunol.* 152, 5368-5374.

18. **Schodin, B. A. and D. M. Kranz**. 1993. Binding Affinity and Inhibitory Properties of a Single-Chain Anti-T Cell Receptor Antibody. *J. Biol. Chem.* 268:25722-25727.

19. **Sha, W. C., C. A. Nelson, R. D. Newbury, D. M. Kranz, J. H. Russell, and D. Y. Loh.** 1988. Selective Expression of an Antigen Receptor on CD8-bearing T Lymphocytes in Transgenic Mice. *Nature* 335, 271-274.

20. **Sha, W. C., C. A. Nelson, R. D. Newberry, D. M. Kranz, J. H. Russell, and D. Y. Loh.** 1988. Positive and Negative Selection of an Antigen Receptor on T Cells in Transgenic Mice. *Nature.* 336:73-76.

21. **Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou.** 1992. RAG-1 Deficient Mice Have No Mature B and T Lymphocytes. *Cell* 68:869-878.

22. **Cho, B.K., B. A. Schodin, and D.M. Kranz.** (1995) Characterization of a Single-Chain Antibody to the β-Chain of the T Cell Receptor. *J. Biol. Chem.* in press.

23. **Sugiyama, Y., M. Aihara, M. Shibamori, K. Deguchi, K. Imagawa, M. Kikuchi, H. Momota, T. Azuma, H. Okada, O. Alper, J. Hitomi, and K. Yamaguchi.** 1992. *In vitro* Anti-tumor Activity of Anti-c-*erb*B-2 x Anti-CD3ε Bifunctional Monoclonal Antibody. *Jpn. J. Cancer Res.* 83:563-567.

24. **Lamoyi, Edmundo.** 1986. Preparation of $F(ab')_2$ Fragments from Mouse IgG of Various Subclasses. *Meth. Enzymol.* 121:652-663.

25. **Stötter, H., E. A. Wiebke, S. Tomita, A. Belldegrun, S. Topalian, S. A. Rosenberg, and M. T. Lotze.** 1989. Cytokines Alter Target Cell Susceptibility to Lysis. II. Evaluation of Tumor Infiltrating Lymphocytes. *J. Immunol.* 142:1767-1773.

26. **Fady, C. A. M. Gardner, J. F. Gera, and A. Lichtenstein.** 1992. Interferon-induced Increase in Sensitivity of Ovarian Cancer Targets to Lysis by Lymphokine-Activated Killer Cells: Selective Effects on HER2/*neu*-overexpressing Cells. *Cancer Res.* 52:764-769.

27. **Shinkai, Y., S. Koyasu, K.-i. Nakayama, K. M. Murphy, D. Y. Loh, E. L. Reinherz, F. W. Alt.** 1993. Restoration of T Cell Development in RAG-2-Deficient Mice by Functional TCR Transgenes. *Science.* 259:822-825.

28. **Janeway, C. and Travers.** 1994. *Immunobiology.* Garland Publishing Inc. New York, NY.1994. 8:20-23.

29. **Weiner, L.M., Holmes, M., Adams, G.P., LaCreta, F.,Watts, P., Garcia de Palazzo, I.** (1993) A Human Tumor Xenograft Model of Therapy with a Bispecific Monoclonal Antibody Targeting c-erbB-2 and CD16. *Cancer Research* 53: 94-100.

30. **Kranz, D.M., T.A. Patrick, K.E. Brigle, M. J. Spinella, and E.J. Roy.** (1995) Conjugates of Folate and Anti-T Cell Receptor Antibodies Specifically Target Folate receptor Positive Tumor Cells for Lysis. *Proc. Natl. Acad. Sci. USA* in press.

Appendix

Two manuscripts that involve this project are provided in the Appendix:

Kranz, D.M., M. Gruber, and E. Wilson. (1995) Properties of Bispecific Single-Chain Antibodies Expressed in *E. coli*. *J. Hematotherapy*, in press.

Kranz, D.M., T.A. Patrick, K.E. Brigle, M. J. Spinella, and E.J. Roy. (1995) Conjugates of Folate and Anti-T Cell Receptor Antibodies Specifically Target Folate receptor Positive Tumor Cells for Lysis. *Proc. Natl. Acad. Sci. USA* in press.

JOURNAL OF HEMATOTHERAPY 4:00–00 (1995) 401–403
Mary Ann Liebert, Inc.

Properties of Bispecific Single Chain Antibodies Expressed in *Escherichia coli*

DAVID M. KRANZ, MEEGAN GRUBER, and ERIK R. WILSON

ABSTRACT

Single chain bispecific antibodies, in which the genes that encode the V_H and V_L regions are linked in tandem, may offer some advantages over other methods of bispecific antibody preparation. To begin to evaluate the potential of this system, a single chain bispecific antibody ($scFv_2$) that binds to the T cell receptor of a cytotoxic T cell clone and to the hapten fluorescein was constructed. The individual scFv regions were joined by a 25 amino acid linker, and the $scFv_2$ protein was obtained from insoluble inclusion bodies after guanidine solubilization and refolding. Fluorescein-purified $scFv_2$ is active at concentrations of ~10 nM (~1 μg/ml) and above in mediating lysis of fluorescein-coupled tumor cells by the cytotoxic T lymphocytes (CTL). This system has now been used to evaluate various features of the $scFv_2$ approach: (a) affinities of the $scFv_2$ for the TCR and fluorescein, (b) yields of the $scFv_2$ from several different purification schemes, (c) relationship of antigen density (i.e., fluorescein density on tumor cells) to the ability to redirect lysis of the tumor cell, and (d) relationship of $scFv_2$ affinity for the tumor antigen to the ability to redirect lysis. This study was performed by using several analogs of fluorescein for which the $scFv_2$ had different affinities. Results showed that it should be possible to select antibodies with appropriate affinities such that tumor cells with typical antigen densities of 10^4–10^5 molecules per cell will be lysed and normal cells that have lower levels of the antigen will be spared.

INTRODUCTION

THREE DIFFERENT GENERAL APPROACHES have been applied to the production of bispecific antibodies (BsAb) (reviewed in 1): (a) secretion from hybrid hybridomas, followed by isolation of the appropriate BsAb from the mixture of "mispaired" immunoglobulins, (b) chemical linkage of two intact or $F(ab')_2$ antibodies with bifunctional cross-linking agents or by disulfide shuffling, and (c) genetic engineering to produce either non-covalently linked BsAb [e.g., diabody (2) and leucine zipper strategies (3)] or covalently linked BsAb [e.g., disulfide linkage of independently expressed antibodies (4) or expression of tandem V regions as a single chain (5)]. One advantage of genetic engineering is the ability to design antibodies that lack C regions and, thus, possible undesirable C region-mediated side effects. Other features, such as increased stability, more rapid and efficient purification strategies, and reduced immunogenicity, can also be engineered in this approach.

In principle, the simplest and currently the smallest version of a BsAb consists of the appropriate V_H and V_L regions from two antibodies linked in frame to form a single chain (5). From a production viewpoint, this construction could represent the ideal form of a BsAb in that it requires fermentation of a single strain and purification of a single species, and it does not involve steps of chemical linkage. Nevertheless, the single chain approach has several significant problems that will need to be overcome. The major problem is associated with refolding of the antibody from insoluble fractions of *Escherichia coli*. Standard refolding procedures generally yield a small

Presented at the IVth International Conference on Bispecific Antibodies in Duck Key, FL, March 1–5, 1995.

The density of fluorescein coupled to tumor cell targets was measured by two independent methods. In the case of FITC-I and Fl-DHPE-labeled cells, ^{125}I-labeled 4-4-20 antibody was used in saturation binding experiments. The same fluorescein-labeled cells were examined by flow cytometry (Coulter Electronics Epics 752), and the fluorescence was compared with fluorescein-labeled calibration beads (Flow Cytometry Standards Corp, San Juan, Puerto Rico). From the number of accessible epitopes determined by the saturation binding curves, a standard curve with the labeled beads was constructed for each experiment and used to determine the number of fluorescein molecules per cell. This approach allowed determination of FITC-II densities, since saturation was difficult to achieve with the ^{125}I-4420 because of the lower affinity. This also provided a more rapid and routine analysis of FITC-I and Fl-DHPE densities. It should be noted that the number of accessible sites indicated probably represents an overestimate because at 37°C, during a cytotoxicity assay, the density of fluorescein molecules is reduced as a result of internalization or shedding.

Cytotoxicity assays

Target cells were incubated with 50 μl (125 μCi) of ^{51}Cr for 1 h at 37°C. After being washed in PBS, cells were coupled with fluorescein as described. Labeled cells were added at 2×10^4 cells per well of a 96-well plate. BsAb preparations were added in complete media, and CTL clone 2C was added at an effector to target cell (E:T) ratio of 5:1. After 4 h at 37°C, supernatant was removed and the percent specific release was calculated (5).

RESULTS AND DISCUSSION

Purification of single chain antifluorescein/anti-T cell receptor antibodies

Our previous work with the scBsAb scFv$_2$1B2/4420 showed that purified antibody could be readily obtained from a fluorescein affinity column at a yield of ~1 mg/L of culture (5). The affinity-purified antibody generally exhibited an IC_{50} in tumor targeting assays that was no better than ~1 μg/ml. This value is at least one to two orders of magnitude higher than one might anticipate in comparison with other forms of BsAb (reviewed in 1). Because the affinity of each monospecific antibody is relatively high (K_d < 10 nM) (10,15), the low efficiency of the scFv$_2$ is likely to be due to the presence of a significant fraction of misfolded antibody, even in the affinity-purified preparation.

With this in mind, we attempted to further purify the properly folded, active form of the scFv$_2$ by ion-exchange chromatography of the affinity-purified antibody. Because we were concerned that ligand eluted material would retain bound ligand, we chose to elute under conditions of high pH, which have yielded consistently active antibody. Eluted material was subjected to anion exchange chromatography with Q-Sepharose. The Q-Sepharose profiles of total protein and antifluorescein activity are shown in Figure 1. The ability of various fractions to target fluorescein-labeled tumor cells was also examined. The results of this analysis and other fluorescein-binding studies (data not shown) showed that only approximately 10% of the fluorescein affinity purified scFv$_2$, eluted at either low or high pH, retains fluorescein-binding activity. This result compares with a monospecific scFv, where greater than 30% of the activity is retained (data not shown). Thus, the relatively low IC_{50} values that are observed with this scFv$_2$ can be attributed in part to the reduced stability of the fluorescein-binding domain after elution.

In addition, only a fraction of the protein from the Q-Sepharose column that is capable of binding fluorescein is active in the tumor targeting assay (Fig. 1). This result implies that a significant portion of the BsAb has not refolded the anti-TCR domain and that the relatively low IC_{50} values that are observed with this scFv$_2$ are also attributed in part to the presence of misfolded anti-TCR V regions.

Nevertheless, the fraction (fraction 42) that was most active in tumor targeting showed a 10-fold increase in activity compared with the affinity-purified forms (Fig. 2). This finding suggests that it may be possible to obtain scBsAb with activities that begin to approach conventional BsAb. Strategies that will increase the folding

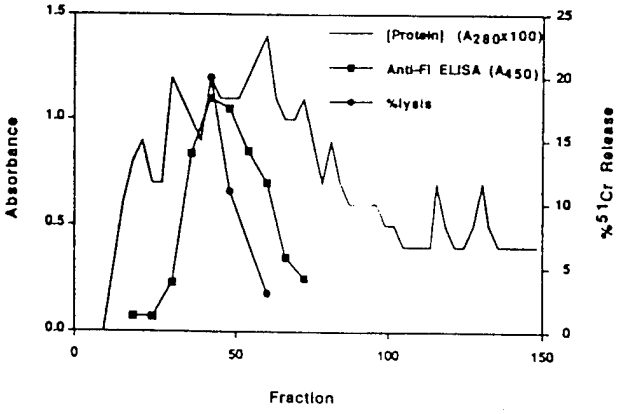


FIG. 1. Q-Sepharose column profile of scBsAb 1B2/4420. scFv$_2$ protein eluted from a fluorescein-sepharose column with 100 mM triethylamine was subjected to anion exchange chromatography through Q-Sepharose and eluted with a 0–1M NaCl gradient. Fractions were monitored for absorbance at 280 nm, for fluorescein binding activity in an ELISA on wells coated with fluorescein-BSA, and for tumor targeting ability in cytotoxicity assays with CTL 2C (5).

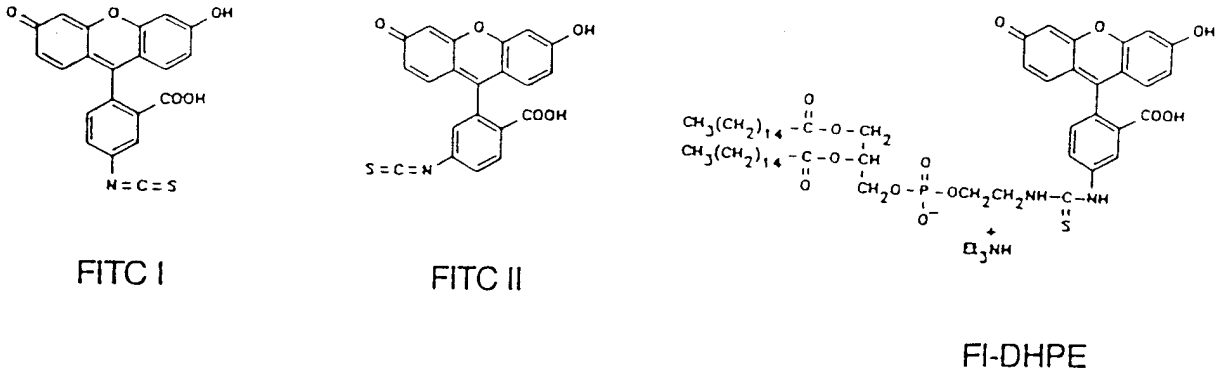


FIG. 3. Structures of fluorescein compounds used as surrogate antigens coupled to tumor cells.

tor cell population. Nevertheless, the results show that it should be possible to select, or engineer, antibodies with appropriate affinities (i.e., affinities that will target tumor cells that bear a particular range of antigen densities but will spare normal cells that might have densities at reduced levels).

ACKNOWLEDGMENTS

We wish to thank Gary Durack of the Flow Cytometry Facility at the University of Illinois for his help in the quantitative flow cytometry. This work was supported by NIH grant AI35990 (to DMK) and grant DAMD17-94-J-4347 from the Department of the Army (to DMK).

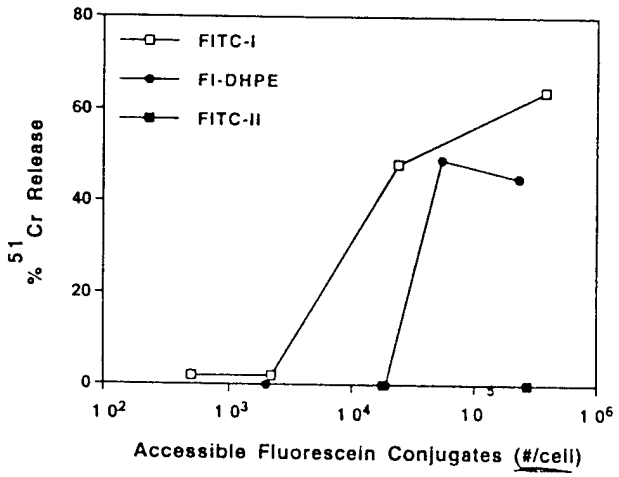


FIG. 4. Effect of antigen density and antibody affinity on ability of bispecific $scFv_2$ to mediate lysis of target cells. Daudi tumor cells were ^{51}Cr-labeled and incubated with various concentrations of FITC-I, FI-DHPE, or FITC-II (see Materials and Methods). The labeled cells were incubated with CTL 2C and BsAb 1B2/4420 in a 4 h ^{51}Cr-release assay. The estimated K_ds of the $scFv_2$ for the ligands were determined to be 1 nM, 6 nM, and 50 nM (calculated at ambient temperature).

REFERENCES

1. Fanger, M.W., Morganelli, P.M. & Guyre, P.M. Bispecific antibodies. Crit. Rev. Immunol. 12:101, 1992.
2. Holliger, P., Prospero, T. & Winter, G. "Diabodies": small bivalent and bispecific antibody fragments. Proc. Natl. Acad. Sci. USA 90:6444, 1993.
3. Kostelny, S.A., Cole, M.S. & Tso, J.Y. Formation of a bispecific antibody by the use of leucine zippers. J. Immunol. 148:1547, 1992.
4. Shalaby, M.R., Shepard, H.M., Presta, L., et al. Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the *HER-2* protooncogene. J. Exp. Med. 175:217, 1992.
5. Gruber, M., Schodin, B., Wilson, E., et al. Efficient tumor cell lysis mediated by a bispecific single-chain antibody expressed in *E. coli*. J. Immunol. 152:5368, 1994.
6. Kranz, D.M. & Voss, E.W. Jr. Restricted reassociation of heavy and light chains from hapten specific monoclonal antibodies. Proc. Natl. Acad. Sci. USA 78:5807, 1981.
7. Mombaerts, P., Iacomini, J., Johnson, R.S., et al. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68:869, 1992.
8. Sha, W.C., Nelson, C.A., Newbury, R.D., et al. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. Nature 335:271, 1988.
9. Kranz, D.M., Tonegawa, S. & Eisen, H.N. Attachment of an anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA 81:7922, 1984.
10. Schodin, B.A. & Kranz, D.M. Binding affinity and inhibitory properties of a single-chain anti-T cell receptor antibody. J. Biol. Chem. 268:25722, 1993.
11. Roehm, N.W., Carbone, A., Kushnir, E., et al. The major histocompatibility complex-restricted antigen receptor on T cells: The genetics of expression of an allotype. J. Immunol. 135:2176, 1985.

Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 000–000, ●●●●●●●● 1995
Immunology

Conjugates of folate and anti-T-cell-receptor antibodies specifically target folate-receptor-positive tumor cells for lysis

DAVID M. KRANZ*, TODD A. PATRICK†, KEVIN E. BRIGLE‡, MICHAEL J. SPINELLA‡, AND EDWARD J. ROY*‡§¶

Departments of *Biochemistry and §Psychology, and †Neuroscience Program, University of Illinois, 600 South Mathews Avenue, Urbana, IL 61801; and ‡Departments of Medicine and the Massey Cancer Center, Virginia Commonwealth University, Medical College of Virginia, Richmond, VA 23298

Communicated by Gregorio Weber, University of Illinois at Champaign-Urbana, Urbana, IL, June 19, 1995

ABSTRACT **High-affinity folate receptors (FRs) are expressed at elevated levels on many human tumors. Bispecific antibodies that bind the FR and the T-cell receptor (TCR) mediate lysis of these tumor cells by cytotoxic T lymphocytes. In this report, conjugates that consist of folate covalently linked to anti-TCR antibodies are shown to be potent in mediating lysis of tumor cells that express either the α or β form of the FR. Intact antibodies with an average of five folates per molecule exhibited high affinity for FR$^+$ tumor cells but did not bind to FR$^-$ tumor cells. Lysis of FR$^+$ cell lines could be detected at concentrations as low as 1 pM (≈0.1 ng/ml), which was 1/1000th the concentration required to detect binding to the FR$^+$ cells. Various FR$^+$ mouse tumor cell lines could be targeted with each of three different anti-TCR antibodies that were tested as conjugates. The antibodies included 1B2, a clonotypic antibody specific for the cytotoxic T cell clone 2C; KJ16, an anti-Vβ8 antibody; and 2C11, an anti-CD3 antibody. These antibodies differ in affinities by up to 100-fold, yet the cytolytic capabilities of the folate/antibody conjugates differed by no more than 10-fold. The reduced size (in comparison with bispecific antibodies) and high affinity of folate conjugates suggest that they may be useful as immunotherapeutic agents in targeting tumors that express folate receptors.**

High-affinity folate receptors (FRs) with a $K_d \approx 1$ nM have recently been detected on the surface of a number of different types of human cancers, particularly ovarian carcinomas and some types of brain tumors (1–4). These receptors differ from the lower affinity reduced-folate/methotrexate (MTX) carriers ($K_d \approx 1$–100 μM) that appear to be largely responsible for the transport of folate-based dihydrofolate reductase inhibitors, such as MTX (5, 6). The presence of FR on human tumor cells has led to the use of FR as a target for specific monoclonal antibodies, such as MOv18 and MOv19 (3). Targeting approaches with monoclonal anti-FR antibodies have included the following: ^{131}I-labeled antibodies (7), engineering of constant regions to optimize antibody-dependent cellular cytotoxicity (8), and bispecific antibodies that target immune effector cells to the FR$^+$ tumor (9–11). The latter studies have used anti-FR antibodies linked to either anti-Fc receptor antibodies or to anti-CD3 antibodies for recruitment of monocytes/natural killer cells or cytotoxic T cells, respectively. Clinical trials with the radiolabeled antibodies and the anti-FR/anti-CD3 bispecific antibodies have recently been initiated (7, 12, 13).

Another approach to targeting FR$^+$ tumor cells has relied on the ability of the FR to endocytose proteins that are covalently linked to folate. For example, Low and colleagues have shown that tumor cells internalize momordin/folate (14) and *Pseudomonas* exotoxin/folate (15) conjugates and liposomes containing chemotherapeutic compounds (16).

Monoclonal antibodies to the mouse FR have not been produced, and, thus, antibody targeting of the FR on mouse tumor cells as a model for human disease has not been possible. Nevertheless, two mouse homologs of the human FR isoforms have been identified, and these receptors also bind folate with high affinity ($K_d \approx 1$ nM) (17, 18). Several mouse leukemia lines have been selected for high FR expression by growth in medium with low concentrations of folate. Two forms (α and β) of the mouse FR have been identified as 38-kDa, lipid-linked membrane glycoproteins (19). As in humans, FRs also appear to be expressed at high levels on some mouse tumors. For example, mouse choroid plexus tumors that arise in mice transgenic for the simian virus 40 large T antigen ●●● (20) express high levels of FR (E.J.R., T.A.P., D.M.K., and T. Van Dyke, unpublished data).

A previous study showed that conjugates of a peptide analog of melanocyte-stimulating hormone and an anti-CD3 antibody mediated lysis of melanoma cells by cytotoxic T lymphocytes (CTLs) (21). The high affinity of FR for folate suggested that attachment of folate directly to an anti-T-cell receptor (TCR) antibody might likewise efficiently target FR$^+$ tumor cells. We show in this report that these conjugates can mediate lysis of the mouse FR$^+$ tumor cells at very low concentrations (≈1 pM). In addition, tumor cell lines with a range of FR densities could be killed, while the parental line with no detectable FR was spared. The effectiveness is likely to be due in part to the finding that the binding affinity of the folate-linked antibody is nearly as high as free folate for the FR α and β forms. This property, together with previous observations that only a few TCRs may need to be engaged to activate a CTL (22), probably accounts for the potency of the folate/anti-TCR conjugates as tumor-targeting agents. Furthermore, the smaller size of the folate/anti-TCR antibody conjugate compared with bispecific antibodies that contain an anti-TCR antibody coupled to an anti-FR antibody may have some therapeutic advantage.

MATERIALS AND METHODS FN1

Cell Lines and Monoclonal Antibodies. The following DBA/2-derived tumor cell lines were maintained in RPMI 1640 medium containing 5 mM Hepes, 10% (vol/vol) fetal bovine serum, 1.3 mM L-glutamine, 50 μM 2-mercaptoethanol, ●●● penicillin, and ●●● streptomycin: Mel, murine erythroleukemia cell (23); La, a subline of Mel selected on low folate (19); L1210, a leukemia cell line (24); LL3, a subline of L1210 selected on low 5-formyltetrahydrofolate (17); and F2-MTX^{r}A, a MTX-resistant L1210 subline selected for increased expression of FRβ by growth on low folic acid (25). La and LL3 express the α form of the folate receptor, and F2-MTX^{r}A expresses the β form of the folate receptor. CTL clone 2C, a mouse alloreactive cell line specific for L^d, was maintained in the same RPMI medium described above and supplemented

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FR, folate receptor; CTL, cytotoxic T lymphocyte; TCR, T-cell receptor; MTX, methotrexate; MHC, major histocompatibility complex; SEB, staphylococcal enterotoxin B.

¶To whom reprint requests should be addressed.

with 10% (vol/vol) supernatant from concanavillin A-stimulated rat spleen cells, 5% α-methyl mannoside, and mitomycin C-treated BALB/c spleen cells as stimulators (26). Monoclonal antibody 1B2, a mouse IgG1 specific for the TCR of CTL 2C, was purified from ascites (27). Hybridoma KJ16, a rat IgG antibody specific for the Vβ8 region of the TCR (28), was cultured in low-serum medium (1% fetal bovine serum in Dulbecco's modified Eagle's medium) in a bioreactor (Amicon), and the antibody was concentrated by ammonium sulfate precipitation. Hybridoma 2C11, a hamster IgG specific for the mouse CD3 ε subunit (29), was cultured in serum-free medium (GIBCO/BRL) and purified over a protein G-Sepharose column. Hybridomas that secrete antibodies to major histocompatibility complex (MHC) class I L^d, 30.5.7 (30), and K^dD^d, 34.1.2s (31), were cultured in the RPMI medium described above and used in flow cytometry without further purification. 30.5.7 was also prepared as ascites fluid and used without further purification in some cytotoxicity assays. Fluorescein-labeled, anti-IgG antibodies were obtained from Kirkegaard & Perry Laboratories.

Polyclonal BALB/c effector cells enriched for activated CTLs were obtained by incubation of 5×10^6 spleen cells per ml with 10 μg of staphylococcal enterotoxin B (SEB) per ml (Toxin Technologies, Madison, WI), 10% (vol/vol) supernatant from concanavillin A-stimulated rat spleen cells, and 5% α-methyl mannoside (32). Cells were used 3 or 4 days after stimulation.

Preparation of Folate/Antibody Conjugates. Folate was coupled through carboxyl groups to antibody amine groups by using a carbodiimide procedure (14). A 5-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Pierce) was added to folate (Sigma) dissolved in dimethyl sulfoxide. After 30 min at room temperature in the dark, a 10- or 100-fold molar excess of the EDC-activated folate was added to 0.5–2.0 mg of antibody in 0.1 M Mops, pH 7.5. After 1 h at room temperature, the sample was applied to a Sephadex G-25 column equilibrated in phosphate-buffered saline (PBS, ●●● pH 7.0). The excluded-peak fractions were pooled and analyzed spectrophotometrically at 280 and 363 nm. Epitope densities of the folate on antibody conjugates were determined by using molar extinction coefficients (ε_M) for folate of 6,197 (363 nm) and 25,820 (280 nm). Antibody concentrations were determined by subtracting the absorbance contribution of folate at 280 nm and by using an antibody ε_M of 224,000. Conjugates were stored at 4°C in the dark.

Mass Spectrometry. Mass spectra were obtained on a TofSpec mass spectrometer by using electrospray ionization. Samples were dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and concentrated to 10–25 pmol/ml. Analysis was performed by the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois.

Folate-Binding Assays. Binding assays were conducted by using ^{125}I-labeled folate (NEN; specific activity = 2200 Ci/mmol; 1 Ci = 37 GBq). Cells were washed with PBS containing 0.1% bovine serum albumin, pH 7.4 (PBS-BSA), to remove excess free folate present in the cell culture medium. Cells, labeled folate, and competitors were incubated in triplicate in 75 μl of PBS-BSA for 1 h. Bound and free ligand were separated by centrifugation through oil [80% (vol/vol) dibutyl phthalate/20% (vol/vol) olive oil] at 12,000 × *g* for 3 sec. Tubes were frozen and cut to allow the radioactivity in the pelleted cells and supernatants to be quantitated separately. Binding parameters were calculated by using LIGAND (33).

Cytotoxicity Assays. Tumor cells were labeled with 50 μl of ^{51}Cr (2.5 mCi/ml) for 60 min at 37°C, washed twice with folate-free RPMI 1640 medium containing 5% (vol/vol) fetal calf serum (folate-free media), and used in 96-well plate cytoxicity assays at 10^4 cells per well. Antibodies and folate/antibody conjugates were added to triplicate wells at various concentrations diluted in folate-free medium. For folate-inhibition studies, folate was added to a final concentration of 2.5 μM. Effector cells (2C or SEB-reactive polyclonal BALB/c T cells) were added at effector-to-target cell ratios of 5:1 or 10:1. For experiments with 2C as the effector cells, the anti-L^d antibody was used at a 1:100 dilution of ascites to inhibit recognition of the L^d alloantigen by CTL 2C. Plates were incubated at 37°C for 4 h, and supernatants were removed for γ counting. Specific ^{51}Cr release was calculated by standard methods. Where indicated the specific release mediated by the folate conjugates was determined by subtracting the release in the absence of the conjugates.

RESULTS

Characterization of Folate/Anti-TCR Antibody Conjugates. The anti-clonotypic antibody 1B2 has a high intrinsic affinity ($K_d \approx 1$ nM) for the TCR on the mouse CTL clone 2C (34). To explore the potential of folate/anti-TCR conjugates, 1B2 was coupled at molar ratios of folate to antibody of 10:1 and 100:1. The 10:1 and 100:1 ratios yielded preparations containing an average molar ratio of 1.3 folates per antibody and 6.0 folates per antibody, respectively.

To confirm these values and to determine the range of epitope densities among the antibodies within a single preparation, the folate/1B2 conjugate (100:1) was examined by electrospray ionization mass spectrometry (Fig. 1). The conjugate had an average molecular mass of 148,935, while the unlabeled 1B2 exhibited a mass of 147,140. By this estimate, the preparation contained an average of 4.1 folates per antibody. Analysis of separate heavy (H) and light (L) chain profiles yielded a value of 4.9 folates per antibody (data not shown). Integration of the mass spectrometry peak indicated that >95% of the antibody molecules contained fewer than 10 folate molecules. F1

Two additional anti-TCR antibodies, KJ16 and 2C11, were also coupled with folate at the 100:1 molar ratio of folate to antibody. These antibodies exhibit different affinities from 1B2, and they recognize TCR epitopes on the Vβ region and CD3 molecules present on mouse CTL 2C (refs. 28, 29, and 35; and Yuri Sykulev and Herman N. Eisen, personal communication). Thus, analysis of these folate/antibody conjugates would allow us to determine if either TCR epitope or antibody affinity affected tumor cell targeting. Each of these preparations exhibited folate densities that were similar to 1B2 (4.9 for KJ16 and 4.4 for 2C11, as judged by spectrophotometry).

Binding of Conjugates to Tumor Cell Lines. To determine if the folate/antibody conjugates bind to the FR on the surface of tumor cells, a competition assay with ^{125}I-labeled folate as the labeled ligand was used. Because cytotoxicity assays are performed at 37°C, all binding studies shown here were also done at 37°C. Previous reports have measured folate binding to FR at 4°C and used a low pH wash to remove endogenously bound folate (19, 36). We determined that conducting the assay at 37°C would allow exchange of labeled and unlabeled

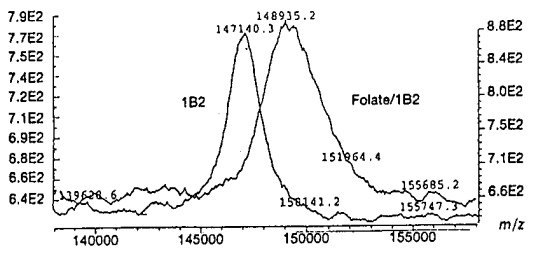


FIG. 1. Mass spectra of anti-TCR antibody 1B2 and folate/1B2 conjugate. The shift in molecular mass of the major peak indicates that an average of approximately four folates are attached to intact 1B2 IgG1.

ligands and produce similar levels of binding compared with acid pretreatment and a 0°C incubation (data not shown). We also determined the affinity of ^{125}I-labeled folate for the FRα and FRβ isoforms of the receptor at 0°C and 37°C by using the FRα^+ and FRβ^+ lines La and F2-MTX^{r}A (Table 1 and Fig. 2 *A* and *B*).

A competition experiment was performed with both cell lines with folate/1B2 (100:1) (Fig. 2 *A* and *B*), folate/1B2 (10:1) (data not shown), and unlabeled 1B2 (data not shown). Both conjugates but not the unlabeled 1B2 inhibited the binding of the labeled ligand. Folate in the conjugated form was about 1/10th as effective at binding than was free folate. This is probably due in part to the fact that carbodiimide coupling can occur through either the α or the γ carboxyl of folate, and only the latter retains binding to FR (15). In addition, it is possible that linkage to some amino groups on the antibody may result in steric hindrance to the FR. Nevertheless, the average K_d of the folate/1B2 preparation for the two cell lines was determined to be 20 nM and 60 nM for FRα and FRβ, respectively.

A comparison of the three different folate/anti-TCR antibody conjugates is shown in Fig. 2*C*. All three conjugates were capable of inhibiting the binding of ^{125}I-labeled folate to FR$^+$ cells. Inhibition was not observed with unlabeled antibodies. The calculated K_d values of the 1B2, KJ16, and 2C11 conjugates in this experiment were 80 nM, 90 nM, and 50 nM, respectively. These similarities indicate that any significant differences in the targeting effectiveness of these antibodies (shown below) are likely to depend on factors other than their folate density.

Conjugates Mediate Specific Lysis of Tumor Cells by a Mouse CTL Clone. To test the targeting efficiency and specificity of the folate conjugates, five different mouse tumor cell lines were examined in a ^{51}Cr-release assay with the mouse CTL clone 2C. Each of these lines was also examined for binding of ^{125}I-labeled folate to approximate the number of FRs at 37°C (Table 1). As expected, the highest levels of expression were detected for the low-folate-selected lines F2-MTX^{r}A, La, and LL3 (19). The parental line L1210 had a low but detectable level of FR, and the parental line Mel had no detectable FR.

Because each of these lines also express the alloantigen L^d that is recognized by CTL clone 2C, assays were performed in the presence of excess monoclonal anti-L^d antibody to minimize non-FR-mediated lysis. As shown in Fig. 3, lysis of each of the FR$^+$ cell lines was detected in the presence of the folate/1B2 conjugate. The lysis was completely inhibited by free folate, indicating that it was mediated by binding to the FR and not by other cell surface molecules—e.g., Fc receptors. The extent of lysis was correlated with the level of surface FR, and the F2-MTX^{r}A line always exhibited more sensitivity than the other lines. In contrast, the FR$^-$ cell line Mel was not lysed, even at a folate/1B2 concentration that was 1000 times higher than the concentration required for detectable killing of the FR$^+$ line F2-MTX^{r}A.

Table 1. Characteristics of cells used as targets for lysis

Cell line	FR type	B_{max}, sites/cell*	K_d, nM 0°	K_d, nM 37°	Class I, mean fluorescent units† L^d	Class I, mean fluorescent units† K^{d}D^d	Class I, mean fluorescent units† No Ab
F2-MTX^{r}A	β	200,000	0.7	5	131	174	4
La	α	60,000	0.9	1	64	76	4
LL3	α	20,000	0.3	ND	144	183	5
L1210	—	8,000‡	ND	ND	125	154	3
Mel	—	<4,000‡	ND	ND	61	79	4

Ab, antibody; ND, not determined.

*Except where indicated, B_{max} was determined from nonlinear regression from the competition curve by using ^{125}I-labeled folate and unlabeled folate.

†MHC class I surface antigens were examined by flow cytometry, as described in *Materials and Methods* ●●●.

‡The levels of values were estimated from a single binding experiment with ^{125}I-labeled folate at 0.7 nM, in comparison with LL3. At 0.7 nM ^{125}I-labeled folate, specific cpm bound per 10^6 cells were 32,000 (F2-MTX^{r}A), 43,000 (La), 15,000 (LL3), 600 (L1210), and <300 (Mel).

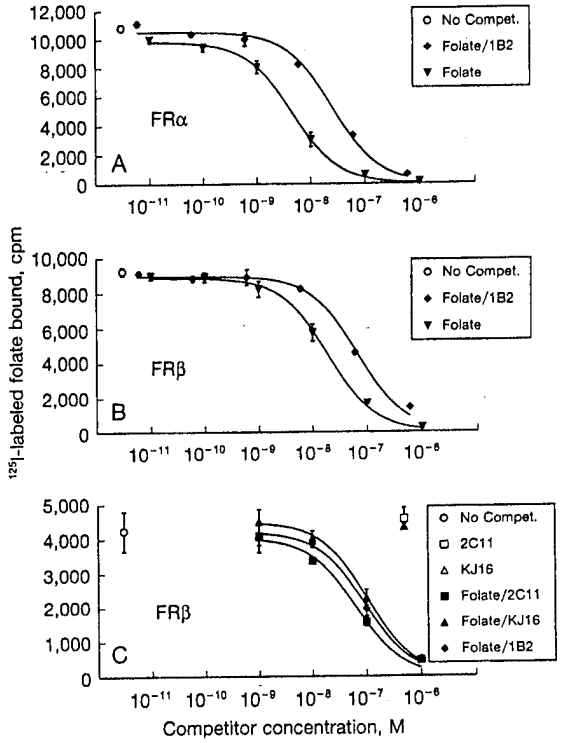


FIG. 2. Inhibition-binding curves of free folate and folate/antibody conjugates. ^{125}I-labeled folate was incubated with two tumor cell types in the presence or absence of competitors for 1 h at 37°C. Concentrations refer to folate rather than antibody concentrations, except in the case of *C*, where unconjugated antibodies were also tested for competition. (*A*) La cells, which express FRα. (*B*) F2-MTX^{r}A cells, which express FRβ. (*C*) Conjugates of folate and three anti-T-cell antibodies were compared in their ability to compete with ^{125}I-labeled folate for binding to F2-MTX^{r}A cells (FRβ). The unconjugated antibodies 2C11 and KJ16 showed no inhibition of ^{125}I-labeled folate binding, as did 1B2 in a separate experiment (data not shown).

To determine if the three different folate/anti-TCR antibody conjugates were all effective at mediating specific lysis, the cell line with the highest FR level, F2-MTX^{r}A (Fig. 4*A*) and the one with lowest—i.e., undetectable—FR level, Mel (Fig. 4*B*) were assayed at various conjugate concentrations. Each of the conjugates mediated lysis of F2-MTX^{r}A but not Mel. The lysis was specific, as indicated by the ability of free folate to inhibit lysis by each of the conjugates.

Conjugates Mediate Specific Lysis of Tumor Cells by Polyclonal CTLs. Activated T cells from a BALB/c mouse were obtained by *in vitro* stimulation of spleen cells with SEB (32). This T-cell population is enriched for Vβ8$^+$/KJ16-reactive cells, although they do not express the epitope of the clonotypic antibody 1B2. The FR$^+$ F2-MTX^{r}A line was efficiently lysed by the BALB/c-derived T cells in the presence of the folate/KJ16 and folate/2C11 conjugates. As expected, lysis was not observed with the folate/1B2 conjugate (Fig. 5*A*). In a separate experiment with polyclonal CTLs, folate/KJ16 and

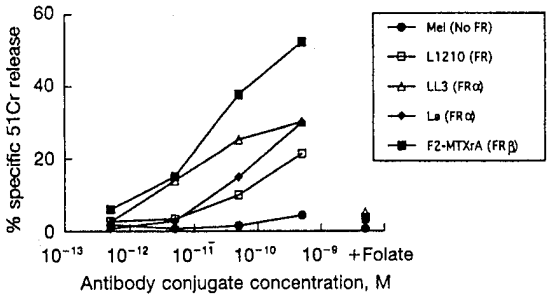


FIG. 3. Cytotoxicity assay of various tumor cell lines with folate/anti-TCR antibody 1B2 and CTL clone 2C. Specific ^{51}Cr release mediated by the indicated concentration of the folate/1B2 conjugate (conjugate with approximately four folates per antibody) was determined in the presence of the anti-L^d antibody to minimize lysis due to recognition of L^d, the nominal ligand for CTL 2C. The ^{51}Cr release measured with 2C and anti-L^d antibody but in the absence of conjugate (0% for Mel, 3% for F2-MTX^{r}A, 0% for La, 37% for L1210, 5% for LL3) was subtracted to yield the values shown. The background values of ^{51}Cr release may have varied between different target cells depending on the level of surface L^d (see Table 1). Assays were incubated for 4 h at an effector-to-target cell ratio of 5:1. Assays with free folate at a final concentration of 2.5 μM were performed with the second highest concentration (0.5 nM) of the folate/1B2 conjugate.

folate/2C11 mediated lysis was shown to be completely inhibited by free folate (data not shown). In contrast, there was relatively little effect on the FR$^-$ line Mel, although at the highest concentration of 2C11, there was some lysis (Fig. 5*B*). This could be due to antibody-dependent cellular cytotoxicity mediated by the conjugate and non-T-cell effectors within the

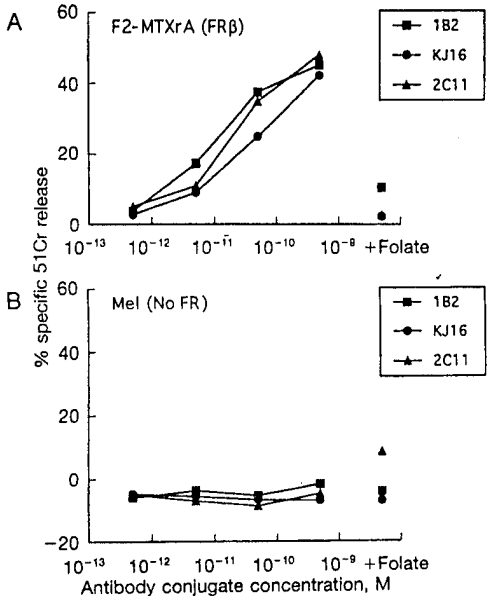


FIG. 4. Cytotoxicity assay of FR$^+$ and FR$^-$ tumor cell lines with three different folate/anti-TCR conjugates and CTL clone 2C. Assays were performed as described in the legend to Fig. 4 at an effector-to-target cell ratio of 10:1. Assays with free folate at a final concentration of 2.5 μM were performed with the highest concentration (0.5 nM) of the folate/1B2 conjugate. (*A*) Lysis of the FR$^+$ cell line F2-MTX^{r}A. ^{51}Cr release with 2C and anti-L^d antibody but in the absence of folate conjugates was 0%. (*B*) Lysis of the FR$^-$ cell line Mel. ^{51}Cr release with 2C and anti-L^d antibody but in the absence of folate conjugates was 0%.

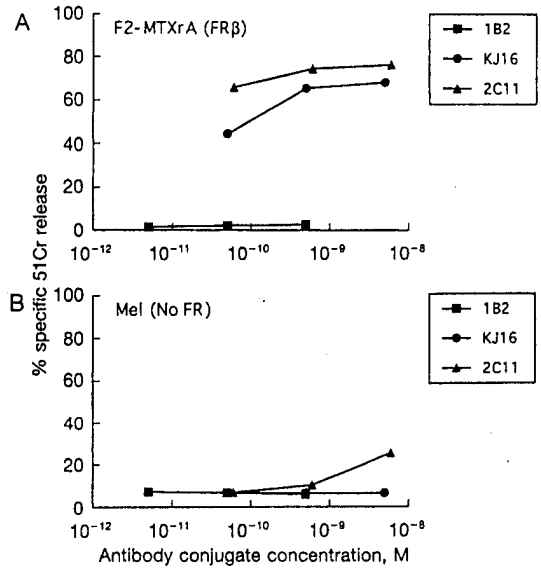


FIG. 5. Cytotoxicity assay of FR$^+$ and FR$^-$ tumor cell lines with three different folate/anti-TCR conjugates and polyclonal BALB/c T cells. SEB-stimulated spleen cells were used as a source of effector cells at an effector-to-target cell ratio of 10:1. (*A*) Lysis of the FR$^+$ cell line F2-MTX^{r}A. ^{51}Cr release with effector cells but in the absence of folate conjugates (27%) was subtracted to yield the indicated values. (*B*) Lysis of the FR$^-$ cell line Mel. ^{51}Cr release with effector cells but in the absence of folate conjugates (17%) was subtracted to yield the indicated values.

polyclonal population. Alternatively, there may be low levels of FR that are not detectable by standard binding assays.

DISCUSSION

Bispecific antibodies that mediate lysis of tumor cells by activated T cells, natural killer cells, monocytes, or macrophages have been shown to be effective in various animal models of disease (reviewed in ref. 37). Monoclonal antibodies to the human FR linked to an anti-CD3 antibody are able to retarget the lysis of these tumor cells *in vitro*. A clinical trial that targets the high-affinity FR present on ovarian tumors with anti-FR × anti-CD3 bispecific antibodies has recently been initiated (7).

In this report, we describe an alternative approach to targeting FR$^+$ tumors for immune-mediated lysis. This approach may offer several advantages over bispecific antibodies. The method takes advantage of the high affinity of folic acid for the FR ($K_d \approx 1$ nM) compared with the affinity of the constitutive reduced folate carrier protein ($K_d \approx 100$ μM) that is expressed by most cells (6). Because the affinity of folate for the FR is of the same order as the highest affinity antibodies, we reasoned that a simple conjugate of folate and the anti-TCR antibody may be as effective as the best bispecific antibodies. Indeed, the folate/anti-TCR conjugates bind to the FR with approximately 1/10th of the affinity compared with free folate, and they mediate specific lysis of the FR$^+$ tumor cells.

Two observations regarding the effectiveness of the folate-targeting approach suggest that it should be generally useful. First, all three different anti-TCR antibodies mediated lysis with less than a ten-fold difference between them, despite the fact that these antibodies differ in affinity by at least 100-fold: 1B2, $K_d \approx 1$ nM (34); KJ16, $K_d \approx 100$ nM (35); 2C11, $K_d > 10$ nM (Yuri Sykulev and Herman N. Eisen, personal communication). Second, four different tumor cell lines with a wide

range of densities of the high-affinity FR were specifically lysed while the FR$^-$ tumor line was spared. The latter result indicates that the ubiquitous expression of folate carrier protein by cells may not result in destruction of most normal cells.

We have noticed that tumor lines with relatively high levels of the FR are not lysed equally well—e.g., F2-MTX^{r}A vs. La; Fig. 2. Clearly there are other factors that contribute to efficient recognition and lysis mediated by the folate/antibody conjugates, just as there are with conventional bispecific antibodies (reviewed in ref. 37). These factors include adhesion molecule levels and intrinsic susceptibilities of the tumor cells. With the F2-MTX^{r}A line, it may be related to the level of MHC class I protein present on this cell compared with the amount on La (Table 1). For example, CD8 on CTL 2C may act synergistically with the TCR by binding to MHC class I on the F2-MTX^{r}A line. If this is the case, then it might be expected that folate/anti-CD8 antibodies will raise the level of killing of the La line to levels observed with the F2-MTX^{r}A line.

It is likely that the folate conjugates described here can be optimized further by engineering antibodies for uniform coupling of folate through the γ-carboxyl. For example, a 10-fold increase in folate/toxin effectiveness was observed when only the γ-carboxyl of folate was coupled through a disulfide bond rather than coupling through carbodiimide-mediated linkages (15). We have previously described the cloning of a single-chain 1B2 antibody (34) and have recently prepared an active single-chain version of KJ16 (Bryan Cho and D.M.K., unpublished data). Jost *et al.* (38) have also described an active anti-CD3 single-chain antibody. Thus, it should soon be possible to create folate/single-chain Fv conjugates and to test their effectiveness in comparison with the intact antibodies described in this report.

The *in vivo* effectiveness of tumor-targeting agents may be influenced by additional factors, such as interactions with serum components, other immune effectors, and normal tissues, but several advantages of the conjugates described here are worth noting. The reduced size and immunogenicity of folate/antibody conjugates should provide some advantage over conventional bispecific antibodies. Folate/antibody conjugates are approximately one-half the size of bispecific antibodies. Thus, folate/single-chain Fv regions will be approximately 30-kDa compared with 60-kDa for the bispecific antibodies which is currently the smallest active form of a bispecific antibody—i.e., two linked Fv regions (39). This reduced size should result in improved tumor penetration and tumor/tissue localization ratios (40). Finally, immunogenicity may be reduced since human anti-immunoglobulin responses to the anti-FR cannot occur when folate is used directly as the targeting moiety.

We thank Drs. ●●● Kappler and ●●● Marrack for the KJ16 hybridoma, and Dr. ●●● Bluestone for the 2C11 hybridoma, Erik Wilson for preparation of the anti-CD3 antibody, Carol Schlueter for providing the anti-L^d ascites fluid, and Dr. I. David Goldman for the DBA/2 tumor cell lines (supported by National Cancer Institute Grants CA39807 and CA09349). Flow cytometry was performed by Gary Durack of the Flow Cytometry Facility and KJ16 antibody was produced by Steve Miklasz of the Immunological Resource Center, both at the University of Illinois. The TofSpec mass spectrometer was purchased in part with Grant RR07141 from the Division of Research Resources, National Institutes of Health. This work was supported by a grant from University of Illinois Research Board (to E.J.R.) and Grant DAMD17-94-J-4347 from the Department of the Army (to D.M.K.).

1. Weitman, S. D., Lark, R. H., Coney, L. R., Fort, D. W., Frasca, V., Zurawski, V. R., Jr., & Kamen, B. A. (1992) *Cancer Res.* **52,** 3396–3401.
2. Ross, J. F., Chaudhuri, P. K. & Ratnam, M. (1994) *Cancer* **73,** 2432–2443.
3. Coney, L. R., Tomassetti, A., Carayannopoulos, L., Frasca, V., Kamen, B. A., Colnaghi, M. I. & Zurawski, V. R., Jr. (1991) *Cancer Res.* **51,** 6125–6132.
4. Weitman, S. D., Frazier, K. M. & Kamen, B. A. (1994) *J. Neurooncol.* **21,** 107–112.
5. Westerhof, G. R., Jansen, G., van Emmerik, N., Kathmann, I., Rijksen, G., Jackman, A. L. & Schornagel, J. H. (1991) *Cancer Res.* **51,** 5507–5513.
6. Goldman, I. D. (1971) *Ann. N.Y. Acad. Sci.* **186,** 400–422.
7. Canevari, S., Miotti, S., Bottero, F., Valota, O. & Colnaghi, M. I. (1993) *Hybridoma* **12,** 501–507.
8. Coney, L. R., Mezzanzanica, D., Sanborn, D., Casalini, P., Colnaghi, M. I. & Zurawski, V. R., Jr. (1994) *Cancer Res.* **54,** 2448–2455.
9. van Ravenswaay Claasen, H. H., van de Griend, R. J., Mezzanzanica, D., Bolhuis, R. L., Warnaar, S. O. & Fleuren, G. J. (1993) *Int. J. Cancer* **55,** 128–136.
10. Mezzanzanica, D., Garrido, M. A., Neblock, D. S., Daddona, P. E., Andrew, S. M., Zurawski, V. R., Jr., Segal, D. M. & Wunderlich, J. R. (1991) *Cancer Res.* **51,** 5716–5721.
11. Ferrini, S., Cambiaggi, A., Cantoni, C., Canevari, S., Mezzanzanica, D., Colnaghi, M. I. & Moretta, L. (1992) *Int. J. Cancer* **7,** 15–18.
12. Bolhuis, R. L. H., Lamers, C. H. J., Goey, H. S., Eggermont, A. M. M., Trimbos, J. B., Stoter, G., Lanzavecchia, A., Di Re, E., Miotti, S., Raspagliesi, F., Rivoltini, L. & Colnaghi, M. I. (1992) *Int. J. Cancer* **7,** 78–81.
13. Lamers, C. H., Gratama, J. W., Warnaar, S. O., Stoter, G. & Bolhuis, R. L. (1995) *Int. J. Cancer* **60,** 450–457.
14. Leamon, C. P. & Low, P. S. (1992) *J. Biol. Chem.* **267,** 24966–24971.
15. Leamon, C. P., Pastan, I. & Low, P. S. (1993) *J. Biol. Chem.* **268,** 24847–24854.
16. Lee, R. J. & Low, P. S. (1995) *Biochim. Biophys. Acta.* **1233,** 134–144.
17. Brigle, K. E., Westin, E. H., Houghton, M. T. & Goldman, I. D. (1991) *J. Biol. Chem.* **266,** 17243–17249.
18. Jansen, G., Schornagel, J. H., Westerhof, G. R., Rijksen, G., Newell, D. R. & Jackman, A. L. (1990) *Cancer Res.* **50,** 7544–7548.
19. Brigle, K. E., Spinella, M. J., Westin, E. H. & Goldman, I. D. (1994) *Biochem. Pharmacol.* **47,** 337–345.
20. Van Dyke, T. A., Finlay, C., Miller, D., Marks, J., Lozano, G. & Levine, A. J. (1987) *J. Virol.* **61,** 2029–2032.
21. Liu, M. A., Nussbaum, S. R. & Eisen, H. N. (1988) *Science* **239,** 395–398.
22. Kageyama, S., Tsomides, T. J., Sykulev, Y. & Eisen, H. N. (1995) *J. Immunol.* **154,** 567–576.
23. Friend, C., Patuleia, M. C. & De Harven, E. (1966) *Natl. Cancer Inst. Monogr.* **22,** 505–522.
24. Law, L. W., B●●●, D. T., Boyle, P. J. & Miller, J. H. (1949) *J. Natl. Cancer Inst.* **10,** 179–192.
25. Brigle, K. E., Seither, R. L., Westin, E. H. & Goldman, I. D. (1994) *J. Biol. Chem.* **269,** 4267–4272.
26. Kranz, D. M., Sherman, D. H., Sitkovsky, M. V., Pasternack, M. S. & Eisen, H. N. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 573–577.
27. Kranz, D. M., Tonegawa, S. & Eisen, H. N. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7922–7926.
28. Haskins, K., Hannum, C., White, J., Rhoem, N., Kubo, R., Kappler, J. & Marrack, K. (1984) *J. Exp. Med.* **160,** 452–471.
29. Leo, O., Foo, M., Sachs, D. H., Samelson, L. E. & Bluestone, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1374–1378.
30. Ozato, K., Hansen, T. H. & Sachs, D. H. (1980) *J. Immunol.* **125,** 2473–2477.
31. Ozato, K., Mayer, N. M. & Sachs, D. H. (1982) *Transplantation* **34,** 113–118.
32. Soo Hoo, W. F. & Kranz, D. M. (1993) *J. Immunol.* **150,** 4331–4337.
33. Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
34. Schodin, B. A. & Kranz, D. M. (1993) *J. Biol. Chem.* **268,** 25722–25727.
35. Rojo, J. M. & Janeway, C. A., Jr. (1988) *J. Immunol.* **140,** 1081–1088.
36. Holm, J., Hansen, S. I., Hoier-Madsen, M. & Bostad, L. (1991) *Biochem. J.* **280,** 267–271.

37. Fanger, M. W., Morganelli, P. M. & Guyre, P. M. (1992) *Crit. Rev. Immunol.* **12,** 101–124.
38. Jost, C. R., Kurucz, I., Jacobus, C. M., Titus, J. A., Geroge, A. J. T. & Segal, D. M. (1994) *J. Biol. Chem.* **269,** 26267–26273.
39. Gruber, M., Schodin, B., Wilson, E. & Kranz, D. M. (1994) *J. Immunol.* **152,** 5368–5374.
40. Yokota, T., Milenic, D. E., Whitlow, M. & Schlom, J. (1992) *Cancer Res.* **52,** 3402–3408.